RECOMBINANT PROTEINS DERIVED FROM HGF AND MSP

Field of the invention

The present invention relates to recombinant proteins obtained from the combination of structural domains derived from the α subunits of hepatocyte growth factor (HGF) and macrophage stimulating protein (MSP).

In particular, the engineered factors of the invention are obtained by combination of the hairpin loop and kringle domains of the α chains of HGF and/or MSP, so as to obtain a structure having two superdomains with an intervening linker sequence. Moreover, the invention relates to DNA sequences encoding the above mentioned recombinant proteins, to the expression vectors comprising said DNA sequences and to host cells containing said expression vectors. The recombinant proteins of the present invention are biologically active, and their activity can be measured by determination of their ability to induce activation of the Met tyrosine kinase receptor, their "scattering" effect on epithelial cells, and their protective effect against cell death induced by chemotherapic drugs (vide infra). Therefore, these molecules can conveniently be used to prevent or treat the toxic side effects of the chemotherapeutical treatment of tumours, and to reduce iatrogenic cell damage induced by other types of drugs.

Technological background

Hepatocyte Growth Factor (HGF) and Macrophage Stimulating Protein (MSP) are highly related proteins both structurally and

functionally (Fig. 1 and 2). Both these factors are secreted as an inactive precursor, which is processed by specific proteases which recognise a cleavage site inside the molecule, dividing the protein in two subunits. These subunits, named α chain and β chain, are linked by a disulphide bond. Thus, the mature factor is an α-β dimeric protein. Only the mature (dimeric) form of the factor is able to activate its receptor at the surface of the target cells (the Met tyrosine kinase in the case of HGF and the Ron tyrosine kinase in the case of MSP) and therefore to mediate biological responses (Naldini, L. et al., 1992, EMBO J. 11: 4825-4833; Wang, M. et al., 1994, J. Biol. Chem. 269; 3436-3440; Bottaro, D. et al., 1991, Science 25: 802-804; Naldini, L. et al., 1991, EMBO J. 10: 2867-2878; Wang, M. et al., 1994, Science 266: 117-119; Gaudino, G. et al., 1994, EMBO J. 13: 3524-3532).

The α chain of both factors contains a hairpin loop (HL) structure and four domains with a tangle-like structure named kringles (K1-K4; Nakamura T et al., 1989, Nature 342:440-443; Han, S. et al.., 1991, Biochemistry 30: 9768-9780). The precursor also contains a signal sequence (LS) of 31 amino acids (in the case of HGF) or of 18 amino acids (in the case of MSP), removed in rough endoplasmic reticulum, which directs the neoformed peptide to the secretive pathway. The β chain contains a box with a sequence homologous to that typical of serine proteases, but it has no catalytic activity (Nakamura T et al., 1989, Nature 342:440-443; Han, S. et al.., 1991, Biochemistry 30: 9768-9780). Both α and β chains contribute to the binding of the growth factor to the respective receptor (Met for HGF and

Ron for MSP).

HGF and MSP polypeptides are able to induce a variety of biological effects besides cell proliferation. The main biological activities of these molecules are: stimulation of cell division (mitogenesis); stimulation of motility (scattering); induction of polarisation and cell differentiation; induction of tubule formation (branched morphogenesis); increase of cell survival (protection from apoptosis). The tissues that respond to HGF and MSP stimulation are those where cells express the respective Met (HGF) and Ron (MSP) receptors. The most important target tissues of these factors are epithelial cells of different organs, such as liver, kidney, lung, breast, pancreas and stomach, and some cells of the hematopoietic and nervous systems. A detailed review of the biological effects of HGF and MSP in the various tissues can be found in Tamagnone, L. & Comoglio, P., 1997, Cytokine & Growth Factor Re-views, 8: 129-142, Elsevier Science Ltd.; Zarnegar, R. & Michalopoulos, G., 1995, J. Cell Biol. 129: 1177-1180; Medico, E. et al., 1996, Mol. Biol. Cell, 7: 495-504; Banu, N. et al., 1996, J. Immunol. 156: S2933-2940.

In the case of HGF, the hairpin loop and the first two kringles are known to contain the sites of direct interaction with the Met receptor (Lokker NA et al., 1992, EMBO J., 11:2503-2510; Lokker, N. et al., 1994, Protein Engineering 7: 895-903). Two naturally-occurring truncated forms of HGF produced by some cells by alternative splicing have been described. The first one comprises the first kringle (NK1-HGF Cioce, V. et al., 1996, J. Biol. Chem.

271: 13110-13115) whereas the second one spans to the second kringle (NK2-HGF Miyazawa, K. et al., 1991, Eur. J. Biochem. 197: 15-22). NK2-HGF induces cell scattering, but it is not mitogenic as the complete growth factor is (Hartmann, G. et al., 1992, Proc. Natl. Acad. Sci. USA 89: 11574-11578). However, NK2-HGF regains mitogenic activity in the presence of heparin, a glucosaminoglycan that binds HGF through a domain contained in the first kringle and which is likely to induce dimerization of NK2-HGF (Schwall, R. et al., 1996, J. Cell Biol. 133: 709-718). Moreover NK2-HGF, being a partial agonist of Met, behaves as a competitive inhibitor of HGF as far as the mitogenic activity is concerned (Chan, A. et al., 1991, Science 254: 1382-1385). NK1-HGF has also been described to exert partial stimulation of Met and competitive inhibition of HGF mitogenic activity (Cioce, V. et al., 1996, J. Biol. Chem. 271: 13110-13115). Anyway, a truncated factor is endowed with an activity markedly lower than the recombinant factors described in the invention, as shown in example 3.

In the case of MSP, the interaction sites with the Ron receptor are less understood: some preliminary studies suggest a situation opposite of that of HGF, i.e. the β chain directly binds the receptor whereas the α chain would act stabilizing the complex (Wang MH et al., 1997, J. Biol. Chem. 272:16999-17004).

The therapeutical use of molecules such as HGF and MSP is potentially valuable in a wide range of pathologies (Abdulla, S., 1997, Mol. Med. Today 3: 233). Nevertheless, a number of technical

as well as biological complications make the application of these molecules in clinics difficult. First of all, the pleiotropic character of these factors can causes poorly selective biological responses, which involve undesired side effects. For example, the use of HGF to prevent some side effects of the chemotherapeutic drug cisplatin has been proposed (Kawaida K. et al., 1994, Proc. Natl. Acad. Sci. 91:4357-4361). Cancer patients treated with this drug can suffer kidney acute damage due to the cytotoxic action of cisplatin on proximal tubule epithelial cells. HGF is able to protect these cells against programmed death (apoptosis) induced by cisplatin, but at the same time it can induce an undesired proliferation of neoplastic cells. Other problems related to the pharmaceutical use of HGF and MSP are the necessity of their proteolytic activation and their stability, which causes technical problems. The NK1 and NK2 truncated forms of HGF do not require proteolytic activation, but they have a reduced biological activity.

Summary of the invention

The present invention provides recombinant molecules composed of a combination of structural domains derived from the α chains of HGF and/or MSP, which overcome the problems of the prior art molecules described above. The molecules of this invention are composed of two superdomains connected by a linker. Each superdomain is composed of a combination of the HL and K1-K4 domains of the α chain of HGF and/or MSP. These engineered factors induce selective biological responses, do not require

proteolytic activation, are stable and are more active than the truncated forms of HGF described previously.

Detailed disclosure of the invention

The present invention relates to recombinant proteins (which will be hereinafter referred to indifferently as proteins, molecules, engineered or recombinant factors) characterised by a structure that comprises two superdomains, each consisting of a combination of HL and K1-K4 domains derived from the α chain of HGF and/or MSP, linked by a spacer sequence or a linker. In particular, the invention relates to proteins of general formula (I)

$$[A] - B - [C] - (D)_y \qquad (I)$$

in which

[A] corresponds to the sequence $(LS)_m$-HL-K1-$(K2)_n$-$(K3)_o$-$(K4)_p$

wherein (the numbering of the following amino acids refers to the HGF and MSP sequences as reported in Fig. 1 and 2, respectively):

LS is an amino acid sequence corresponding to residues 1-31 of HGF or 1-18 of MSP;

HL is an amino acid sequence derived from the α chain of HGF starting between residues 32-70 and ending between residues 96-127; or it is an amino acid sequence derived from the α chain of MSP starting between residues 19-56 and ending between residues 78-109;

K1 is an amino acid sequence derived from the α chain of HGF starting between residues 97-128 and ending between residues 201-205; or it is an amino acid sequence derived from the α chain of MSP starting between residues 79-110 and ending between residues 186-190;

K2 is an amino acid sequence derived from the α chain of HGF starting between residues 202-206 and ending between residues 283-299; or it is an amino acid sequence derived from the α chain of MSP starting between residues 187-191 and ending between residues 268-282;

K3 is an amino acid sequence derived from the α chain of HGF starting between residues 284-300 and ending between residues 378-385; or it is an amino acid sequence derived from the α chain of MSP starting between residues 269-283 and ending between residues 361-369;

K4 is an amino acid sequence derived from the α chain of HGF starting between residues 379-386 and ending between residues 464-487; or it is an amino acid sequence derived from the α chain of MSP starting between residues 362-370 and ending between residues 448-481;

m, n, o, p can be 0 or 1;

the sum $n + o + p$ is an integer from 1 to 3 or 0, with the proviso that $n \geq o \geq p$;

B is the sequence $[(X)_q\ Y]_r$, wherein X = Gly and Y = Ser, or Cys, or Met, or Ala;

q is an integer from 2 to 8;

r is an integer from 1 to 9;

[C] corresponds to the sequence HL-K1-$(K2)_s$-$(K3)_t$-$(K4)_u$

wherein HL, K1-K4 are as defined above,

s, t, u are 0 or 1; the sum $s + t + u$ is an integer from 1 to 3 or 0, with the proviso that $s \geq t \geq u$;

D is the sequence W-Z, wherein W is a conventional proteolytic site, Z is any tag sequence useful for the purification and detection of the protein; y

is 0 or 1.

Non-limiting examples of W are consensus sequences for enterokinase protease, thrombin, factor Xa and IgA protease.

Preferred proteins of general formula (I), are those in which: the HL domain is a sequence of HGF α chain ranging from amino acids 32 to 127, or a sequence of MPS α chain ranging from amino acids 19 to 98; the K1 domain is a sequence of HGF α chain ranging from amino acids 128 to 203, or a sequence of MPS α chain ranging from amino acids 99 to 188; the K2`domain is a sequence of HGF α chain ranging from amino acids 204 to 294, or a sequence of MPS α chain ranging from amino acids 189 to 274; the K3 domain is a sequence of HGF α chain ranging from amino acids 286 to 383, or a sequence of MPS α chain ranging from amino acids 275 and 367; the K4 domain is a sequence of HGF α chain ranging from amino acids 384 to 487, or a sequence of MPS α chain ranging from amino acids 368 and 477.

Among the possible combinations of the domains of general formula (I), the following (II) and (III) are preferred, concerning two recombinant factors named Metron Factor-1 and Magic Factor-1, respectively:

LS_{MSP}-HL_{MSP}-$K1_{MSP}$-$K2_{MSP}$-L-HL_{HGF}-$K1_{HGF}$-$K2_{HGF}$-D (Metron Factor-1)

(II)

and

LS_{HGF}-HL_{HGF}-$K1_{HGF}$-$K2_{HGF}$-L-HL_{HGF}-$K1_{HGF}$-$K2_{HGF}$-D (Magic Factor-1)

(III)

For both molecules, L is a linker sequence $(Gly_4Ser)_3$, D is a tag

sequence Asp_4-Lys-His_6.

For Metron Factor-1, LS_{MSP} is the sequence 1-18 of MSP, HL_{MSP} is the sequence 19-56 of MSP, $K1_{MSP}$ is the sequence 99-188 of MSP, $K2_{MSP}$ is the sequence 189-274 of MSP, HL_{HGF} is the sequence 32-127 of HGF, $K1_{HGF}$ is the sequence 128-203 of HGF, $K2_{HGF}$ is the sequence 204-294 of HGF.

For Magic Factor-1, HL_{HGF}, $K1_{HGF}$, $K2_{HGF}$ are as defined above, LS_{HGF} is the sequence 1-31 of HGF.

The hybrid molecules of the invention are prepared by genetic engineering techniques according to a strategy involving the following steps:

a) construction of DNA encoding the desired protein;

b) insertion of DNA in an expression vector;

c) transformation of a host cell with recombinant DNA (rDNA);

d) culture of the transformed host cell so as to express the recombinant protein;

e) extraction and purification of the produced recombinant protein.

The DNA sequences corresponding to HGF or MSP structural domains can be obtained by synthesis or starting from DNA encoding for the two natural factors. For example, screening of cDNA libraries can be carried out using suitable probes, so as to isolate HGF or MSP cDNA. Alternatively, HGF or MSP cDNA can be obtained by reverse transcription from purified mRNA from suitable cells.

cDNAs coding for the fragments of HGF and MSP β chains can be amplificated by PCR (Mullis, K.B. and Faloona, F.A., 1987, Methods in

Enzymol. 155, 335-350), and the amplification products can be recombined making use of suitable restriction sites, naturally occurring in the factor sequences or artificially introduced in the oligonucleotide sequence used for the amplification.

In greater detail, one of the above mentioned strategies can be the following:

the portions of DNA encoding the LS, HL, K1, K2, K3 and K4 domains are amplificated by PCR from HGF or MSP cDNA and then recombined to obtain the hybrid sequences corresponding to [A] and [C]. Oligonucleotides recognising sequences located at the two ends of the domains to be amplificated are used as primers. Primers are designed so as to contain a sequence allowing recombination between the DNA of a domain and the adjacent one. Said recombination can be carried out by endonuclease cleavage and subsequent ligase reaction, or making use of the recombinant PCR method (Innis, NA et al.., 1990, in PCR Protocols, Academic Press, 177-183).

The sequence encoding the domain B (linker) can be obtained by synthesis of a double chain oligonucleotide, which can be inserted between [A] and [C] using suitable restriction sites.

The resulting three fragments encoding for [A], [B] and [C] are then inserted in the correct sequence in a suitable vector. In this step it can be decided whether to add or not the domain D (tag), obtained by synthesis analogously to domain B, downstream fragment [C].

The recombinant expression vector can contain, in addition to the recombinant construct, a promoter, a ribosome binding site, an initiation

codon, a stop codon, optionally a consensus site for expression enhancers.

The vector can also comprise a selection marker for isolating the host cells containing the DNA construct. Yeast or bacteria plasmids, such as plasmids suitable for Escherichia Coli, can be used as vectors, as well as bacteriophages, viruses, retroviruses, or DNA.

The vectors are cloned preferably in bacterial cells, for example in Escherichia Coli, as described in Sambrook J., 1989, Molecular Cloning, Cold Spring Harbor Laboratory Press, New York, and the colonies can be selected, for example, by hybridisation with radiolabelled oligonucleotide probes; subsequently, the rDNA sequence extracted from the positive colonies is determined by known methods.

The vector with the recombinant construct can be introduced in the host cell according to the competent cell method, the protoplast method, the calcium phosphate method, the DEAE-dextran method, the electric impulses method, the in vitro packaging method, the viral vector method, the micro-injection method, or other suitable techniques.

Host cells can be prokaryotic or eukaryotic, such as bacteria, yeasts or mammal cells, and they will be such as to effectively produce the recombinant protein.

After transformation, cells are grown in a suitable medium, which can be for example MEM, DMEM or RPMI 1640 in the case of mammal host cells.

The recombinant protein is secreted in the culture medium from which it can be recovered and purified with different methods, such as mass exclusion, absorption, affinity chromatography, salting-out, precipitation, dialysis, ultrafiltration.

A simple, rapid system for the production of the molecules of the invention is, for example, transient expression in mammal cells.

Accordingly, the plasmid containing the recombinant DNA fragment, for example PMT2 (Sambrook, J. et al., 1989, Molecular Cloning, Cold Spring Harbor Laboratory Press), is transfected in suitable recipient cells, such as Cos7 (Sambrook, J. et al., supra) by the calcium phosphate technique or other equivalent techniques. Some days after transfection, the conditioned medium of the transfected cells is collected, cleared by centrifugation and analysed for its content in factor. For this analysis, antibodies directed against HGF or MSP, or against any tag sequence, can be used: the supernatant is immunoprecipitated and then analysed by western blot with the same antibody. The supernatant containing the recombinant factor can also be used directly for biochemical and biological tests. The protein can be purified, for example, using a poly-histidine tag sequence, by absorption on a nickel resin column and subsequent elution with imidazole.

The biochemical properties of the recombinant factors of the invention were tested in connection with their ability to activate Met and Ron receptors.

Sub-micromolar concentrations of the factors have proved to induce phosphorylation in Met tyrosine in human epithelial cells A549, whereas

they do not induce phosphorylation above basal values in cells expressing Ron. On the whole, the tests proved that the first two kringles of HGF maintain their ability to interact and to activate Met tyrosine kinase receptor, whereas the corresponding first two kringles of MSP are not sufficient for modulating the catalytic activity of the Ron receptor. However, the interaction with Ron, although at low affinity, can contribute to the recruitment of the factor at the cell surface, playing a similar role to low affinity receptors (of mature glycoprotein) which recruit the HGF intact molecule through the heparin-binding domain.

The molecules of the invention have a marked biological activity, measured by the scattering tests, and a protecting activity against cell apoptosis induced by cisplatin or etoposide.

In particular, the supernatant containing the recombinant factor has been found to promote scattering of epithelial cells of various nature even at nanomolar concentrations. In these tests, kidney epithelial cells (MDCK) or hepatocyte precursors (MLP29) were used.

In an in vitro experimental system, in which DNA fragmentation typical of apoptotic cells is evaluated by the TUNEL method (Gavrieli, Y. et al., 1992, J. Cell. Biol. 117, 493-501), the recombinant factors protect against apoptosis induced by chemotherapeutic drugs at levels comparable with HGF and remarkably higher than MSP. The engineered molecules proved to be active on human primary epithelial cells from proximal tubule (PTECs), on an immortalised PTECs line (Loc) and on the already cited murine hepatocytes MLP29.

Among the applications of the recombinant molecules of the

invention, the following can be cited:

- prevention of myelotoxicity; in particular they can be used for the expansion of marrow precursors, to increase proliferation of the hematopoietic precursors or to stimulate their entry in circle;
- prevention of liver and kidney toxicity, and of mucositis following antineoplastic treatments; in particular the recombinant factors can be used to prevent toxicity (apoptosis) on differentiated cell elements of liver, kidney and mucosa of the gastroenteral tract, and to stimulate staminal elements of cutis and mucosas to allow the regeneration of germinative layers;
- prevention of chemotherapeutic neurotoxicity.

In general, the proteins of the invention provide the following advantages, compared with the parent molecules HGF and MSP:

- they are smaller molecules with a more compact structure;
- they are more stable and are produced in higher amounts;
- they require no endoproteolytic cleavage for activation, which transforms the HGF and MSP precursors into the respective active forms;
- they can be engineered in combinations of different functional domains, thereby modulating the biological effects, increasing the favourable ones and reducing those undesired (for example, protection from apoptosis versus cell proliferation).

The invention has to be considered also directed at amino acid and nucleotide sequences referred to formula (I), having modifications which can, for example, derive from degeneration of genetic code, without

therefore modifying the amino acid sequence, or from the deletion, substitution, insertion, inversion or addition of nucleotides and/or bases according to all the possible methods known in the art.

Furthermore, the invention relates to the expression vectors comprising a sequence encoding for a protein of general formula (I), which can be plasmids, bacteriophages, viruses, retroviruses, or others, and to host cells containing said expression vectors.

Finally, the invention relates to the use of the recombinant proteins as therapeutical agents, and to pharmaceutical compositions containing an effective amount of the recombinant proteins together with pharmacologically acceptable excipients.

Description of the Figures

(In the following legends, -His located after the name of the parent factors, truncated or recombinant, or of the plasmids, means that the respective sequences contain a poly-histidine tag).

Figure 1:

a) Nucleotide and amino acid sequence of human HGF (Gene Bank # M73239; Weidner, K.M., et al., 1991, Proc. Acad. Sci. USA, 88:7001-7005). In contrast to the cited reference, in the numbering used herein, nucleotide No. 1 is the first base of the initiation codon (the A of the first ATG). The first amino acid is the corresponding methionine. The cDNA untranslated regions at 5' and 3' are neither represented nor considered in the numbering.

b) Nucleotide and amino acid sequence of human MSP (Gene Bank # L11924; Yoshimura, T., et al., 1993, J. Biol. Chem., 268:15461-

15468). In contrast to the cited reference, in the numbering used herein nucleotide No. 1 is the first base of the initiation codon (the A of the first ATG). The first amino acid is the corresponding methionine. The cDNA untranslated regions at 5' and 3' are neither represented nor considered in the numbering.

Figure 2:

a) Molecular structure of Metron Factor-1. The leader sequence is removed from the cells used for the production before secretion and is therefore absent in the mature molecule. The poly-histidine tag can be removed by digestion with the protease enterokinase.

b) Nucleotide and amino acid sequence of Metron-Factor-1. The nucleotide sequence starts with the EcoRI site and terminates with the SalI site (first six bases and last six bases, respectively). The initiation codon (ATG) and the stop codon (TAG) are underlined.

Figure 3:

a) Molecular structure of Magic Factor-1. The leader sequence is removed from the cells used for the production before secretion and is therefore absent in the mature molecule. Poly-histidine tag can be removed by digestion with the protease enterokinase.

b) Nucleotide and amino acid sequence of Magic Factor-1. The nucleotide sequence starts with the SalI site (first six bases and last six bases, respectively). The initiation codon (ATG) and the stop codon (TAG) are underlined.

Figure 4:

Production of Metron-F-1 by transient transfection of mammal

cells. The conditioned supernatants from BOSC cells transfected with the control plasmid (CTRL) or with pRK7-Metron F-1-His were immunoprecipitated with an anti-MSP polyclonal antibody and detected by western blot with the same antibody.

Figure 5:

Quantitation of the recombinant proteins by western blot. (A) The proteins were absorbed on Sepharose-A-heparin beads and detected with an anti-poly-histidine monoclonal antibody. (B) The proteins were immunoprecipitated with an anti-MSP polyclonal antibody and detected with an anti-poly-histidine monoclonal antibody.

Figure 6:

Scattering test carried out on kidney epithelial cells (MDCK) using the recombinant proteins prepared by transient transfection. The protein content was quantified by western blot (see Fig. 5). (A) non-stimulated cells; (B) cells stimulated with control supernatant; (C) cells stimulated with HGF-His; (D) cells stimulated with NK2-HGF-His; (E) cells stimulated with Metron Factor-1; (F) cells stimulated with Magic Factor-1.

Figure 7:

Activation (phosphorylation) of Met receptor by the hybrid factor Metron Factor-1. Human epithelial cells (A549) were stimulated with supernatants conditioned from BOSC cells transfected with the control plasmid (CTRL) or with pRK7-Metron-F-1-His (METRON F-1) at the indicated dilutions. Cell lysates from the stimulated cells were immunoprecipitated with an anti-Met monoclonal antibody and detected by western blot with an anti-phosphotyrosine monoclonal antibody.

Figure 8:

Protective effect of Metron-F-1 against acute renal failure induced by $HgCl_2$ in vivo. Balb-c mice were injected i.v. with Metron-F-1 or vehicle at 0.5 h before and 6, 12, 24, 36 and 48 h after $HgCl_2$ i.v. administration. BUN and histological evaluation of renal necrosis were measured at 72 h.

Data expressed as mean + e.s. of 7 animals/group (BUN) or 3 animals/group (histology).

The following examples illustrate in greater detail the invention.

Example 1a: Preparation of the recombinant construct encoding Metron Factor-1

HGF cDNA was obtained by the RT-PCR technique (Reverse Transcriptase PCR; in: Innis, M. A., et al.., 1990, PCR Protocols, Academic Press, 21-27) from a human lung fibroblast cell line (MRC5; Naldini, L. et al., 1991, EMBO J. 10: 2867-2878). MSP cDNA was obtained with the same technique from human liver (Gaudino, G., et al., 1994, EMBO J. 13: 3524-3532).

The fragment corresponding to MSP LS-HL-K1-K2 was amplified by PCR using MSP cDNA as template and the following oligonucleotides as primers:

P1 (sense)

5' CGCGCGGAATTCCACCATGGGGTGGCTCCCACTCCT 3'

P2 (antisense)

5' CGCGCGCTCGAGGCGGGGCTGTGCCTCGGACCCGCA 3'

in which the underlined palindromic sequences are the restriction sites for

sub B5 Cont'd

the enzymes EcoRI (oligonucleotide P1) and XhoI (oligonucleotide P2). The PCR product was digested with the restriction enzymes EcoRI and XhoI and then purified by electrophoresis on agarose gel.

The fragment corresponding to HL-K1-K2 of HGF was amplified by PCR using HGF cDNA as template and the following oligonucleotides as primers:

sub B6

P3 (sense)

5' CGCGCGTCTAGAGGGACAAAGGAAAAGAAGAAATAC 3'

P4 (antisense)

5' CGCGCGAAGCTTTGTCAGCGCATGTTTTAATTGCAC 3'

in which the underlined palindromic sequences are the restriction sites for the enzymes XbaI (oligonucleotide P3) and HindIII (oligonucleotide P4). The PCR product was digested with the restriction enzymes XbaI and HindIII and then purified by electrophoresis on agarose gel.

For the linker sequence, the following partially complementary oligonucleotides were synthesised, and were subsequently annealed to obtain a double strand DNA fragment with sticky ends:

P5 (sense)

sub B7

5'TCGAGGGCGGTGGCGGTTCTGGTGGCGGTGGCTCCGGCGGTGGCGGTTCT 3'

P6 (antisense)

5'CTAGAGAACCGCCACCGCCGGAGCCACCGCCACCAGAACCGCCACCGCCC 3'

in which the underlined bases are the sequences compatible with the restriction sites for the enzymes XhoI (oligonucleotide P5) and XbaI (oligonucleotide P6).

The resulting three DNA fragments were subcloned in the EcoRI-

HindIII sites of the expression vector pRK7 (Gaudino, G., et al., 1994, EMBO J. 13: 3524-3532), to obtain the recombinant plasmid pRK7-Metron-F-1, containing all the components of Metron Factor-1 except the tag sequence.

For the insertion of the tag sequence, the following partially complementary oligonucleotides were synthesised, and were subsequently annealed to obtain a double strand DNA fragment with sticky ends:

P7 (sense)

5' AGCTGACGACGACGACAAACACCACCACCACCACCACCACTAGGGTCGAC 3'

P8 (antisense)

5' AGCTGTCGACCCTAGTGGTGGTGGTGGTGGTGTTTGTCGTCGTCGTC 3'

in which the underlined bases are compatible with the HindIII restriction site and the boxed palindromic sequences are the consensus sequences for the enzyme SalI. The resulting double strand DNA fragment was inserted in the restriction site HindIII of the recombinant plasmid obtained at the previous step (destroying the HindIII site and creating the SalI site), to obtain the plasmid pRK7-Metron-F-1-His.

Example 1b: Production of Metron Factor-1

The expression vector pRK7 contains a promoter of human cytomegalovirus immediate-early gene (CMV) and an episomal replication origin site of the DNA virus SV40. Therefore, this plasmid is particularly suitable for the expression of proteins in cells expressing the large T antigen of the virus SV40, such as kidney epithelial BOSC cells (Sambrook, J. et al.., 1989, Molecular Cloning, Cold Spring Harbor Laboratory Press). Metron Factor-1 can then be produced by transient

transfection of plasmid pRK7-Metron F-1-His in BOSC cells.

For transfection, 10^6 cells are seeded at day 0 in a 100 mm plate in 90% Dulbecco's Modified Eagle Medium (DMEM)-10% bovine calf serum (10 ml/plate). At day 1, cells are transfected with 10 µg/plate of pRK7-Metron-F-1-His by lipofection, using the protocol provided by the lipofectin producer (Gibco-BRL). At day 2, the DNA-containing medium is substituted by fresh medium with low content in serum (99.5% DMEM-0.5% bovine calf serum). At day 4 (48 hours after the end of the transfection), the medium is collected, cleared by centrifugation, and analysed for its content in Metron Factor-1.

This analysis can be carried out in different ways. For example, the recombinant protein present in the cleared supernatant can be immunoprecipitated with an anti-MSP antibody and then detected by western blot with the same antibody (Fig. 4). In the example shown in figure 4, 500 µl of supernatant (cleared by centrifugation, buffered in 25 mM HEPES and added with a protease inhibitors cocktail) were immunoprecipitated (2 hours at 4° C) with 20 µl of Sepharose-A beads (Pharmacia) covalently conjugated with 2 µl of anti-MSP polyclonal antibody. The beads pellet was washed 3 times with 500 µl of washing buffer (20 mM HEPES pH 7.4; 150 mM NaCl; 0.1% Triton X-100; 10% glycerol) and heated at 90° C for 2 minutes in 100 µl of Laemmli buffer. Eluted proteins were separated by SDS-PAGE on 8% BIS-acrylamide gel, transferred onto membrane (Hybond-C; Amersham) and analysed by western blot. For this analysis, the same rabbit serum used for immunoprecipitation was employed as primary antibody with a 1:1000

dilution and protein A conjugated with peroxidase (Amersham) was used as secondary antibody. Protein A was detected by ECL (Amersham) following the protocol provided by the producer.

Alternatively, the recombinant protein can be partially purified by adsorption on Sepharose-A beads conjugated with heparin and subsequent analysis by western blot using antibodies directed to poly-histidine tag (Fig. 5).

In the example shown in figure 5, the Sepharose-A-heparin beads (20 µl; Pierce) were incubated (4 hours at 4° C) with 500 µl of supernatant (cleared by centrifugation, buffered in 25 mM HEPES and added with a protease inhibitors cocktail) in the presence of 500 mM NaCl, washed with suitable buffer (500 mM NaCl; 20 mM HEPES pH 7.4; 0.1% Triton X-100; 10% glycerol) and heated at 90° C for 2 minutes in 100 µl Laemmli buffer. Eluted proteins were separated by SDS-PAGE on 8% bis-acrylamide gel, transferred onto membrane (Hybond-C; Amersham) and analysed by western blot. For this analysis, a mouse monoclonal antibody to poly-histidine (Invitrogen) diluted 1:5000 was used as primary antibody and an anti-mouse IgG ovine antibody conjugated with peroxidase (Amersham) was used as secondary antibody. The secondary antibody was detected by ECL (Amersham) following the protocol provided by the producer.

The procedure of adsorption on heparin beads can also be used as protocol for the semi-purification of the recombinant protein. Furthermore, the molecule can additionally be purified making use of the poly-histidine affinity to heavy metals such as nickel. The protein containing poly-

histidine tag can be adsorbed on a nickel resin column (Invitrogen) and subsequently eluted with imidazole (the detailed protocol is provided by the manufacturer).

Example 1c: METRON-F-1 production in insect cells

The cDNA encoding for Metron-F1 was subcloned in a suitable expression vector (p-FASTBAC) to generate a recombinant plasmid containing the Metron-F1 gene (p-FASTBAC-Metron). A competent E. Coli strain (DH10 Bac) was transformed with p-FASTBAC-Metron to generate BACMID DNA. The DNA of positive colonies was isolated and checked by PCR to show the correct integration of the expression vector. Subsequently, the DNA from three clones was transfected into Sf9 insect cells with CellFECTIN reagent to produce virus particles. Virus titer was tested by a plaque assay. Single plaques were isolated and used for further propagation of the baculovirus. Viral stock was subsequently expanded in insect cells to scale up METRON-F-1 production. To verify protein expression, insect cells were infected with a multiplicity of infection (MOI) of 1 in a small-scale reactor. Samples of supernatants were analysed by SDS-PAGE followed by western blotting.

To produce amounts adequate for in vivo testing, insect cells were propagated in a 2.5-Liter stirred tank bioreactor. Cells were grown to a cell density of 1.106 ml^{-1} before they were infected with a MOI of 1. Cell suspension was harvested 3 days post infection. The supernatant containing the recombinant protein was separated by centrifugation. The presence of Metron F-1 in the supernatant was proved by SDS-PAGE followed by western blotting. Metron F-1 was pre-purified by a dual step

affinity chromatography on heparin sepharose (heparin-Hi Trap, Pharmacia) at 6° C. For in vivo testing or for further purification steps, the eluted fractions containing Metron F-1 were desalted by Sephadex G-25 chromatography (PD-10 or HiPrep 26/10, Pharmacia). Metron F-1 was further purified by chromatography on HisTrap columns (Pharmacia) and eluted by an imidazole gradient (0-0.5 M) using either a low-pressure system (Econo System, BIO-RAD) or an FPLC system (Pharmacia). Metron F-1 was eluted at an imidazole concentration of about 0.15 M. For in vivo testing, the eluted fractions containing Metron F-1 were freed of imidazole by Sephadex G-25 chromatography as already described, using the buffer to be used for animal treatment.

Example 2a: Preparation of the recombinant construct encoding for Magic Factor-1

HGF cDNA and the plasmid pRK7-Metron-F-1-His described above were used as starting DNA. The fragment corresponding to LS-HL-K1-K2 of HGF was amplificated by PCR using HGF cDNA as template and the following oligonucleotides as primers:

P9 (sense)

5' CGCGCGGGATCCGCCAGCCCGTCCAGCAGCACCATG 3'

P10 (antisense)

5' CGCGCGAAGCTTTGTCAGCGCATGTTTTAATTGCAC 3'

in which the underlined palindromic sequences are the restriction sites for the enzymes BamHI (oligonucleotide P9) and HindIII (oligonucleotide P10). The PCR product was digested with the restriction enzymes BamHI and HindIII and then purified by electrophoresis on agarose gel.

Pub B9

25

For the linker, the following partially complementary oligonucleotides were synthesized, and subsequently annealed to obtain a double strand DNA fragment with sticky ends:

P11 (sense)

5'AGCTTCGGGCGGTGGCGGTTCTGGTGGCGGTGGCTCCGGCGGTGGCGGTTCT3'

P12 (antisense)

5'CTAGAGAACCGCCACCGCCGGAGCCACCGCCACCAGAACCGCCACCGCCCGA3'

in which the underlined bases are the sequences compatible with the restriction sites for the enzymes HindIII (oligonucleotide P11) and XbaI (oligonucleotide P12). The fragment resulting by PCR and the double strand linker sequence were inserted in the plasmid pRK7-Metron-F-1-His in place of the fragment EcoRI-XbaI by means of an EcoRI-BamHI adapter, to obtain the plasmid pRK7-Magic-F-1-His.

Example 2b: Production of Magic Factor-1

Magic Factor-1 is produced on a small scale by transient transfection of BOSC cells analogously to what described for Metron Factor-1. Semi-purification is performed by adsorption on Sepharose-A beads conjugated with heparin followed by Western blot analysis using anti-poly-histidine antibodies (Fig. 5).

Example 3: Biological activity (scattering) on epithelial cells.

The biological activity of recombinant HGF, NK2-HGF, Metron Factor-1 and Magic Factor-1 was tested by a "scatter" assay on MDCK epithelial cells. For this functional test, cells are plated at day 0 in 96-well plates (10^3 cells/well) in 90% DMEM - 10% bovine calf serum. At day 1 the medium is substituted with fresh medium buffered with 50 mM

HEPES pH 7.4 and the supernatant containing the recombinant protein is added at different dilutions. At day 2 cells are washed with DPBS (Dulbecco's Phosphate Buffered Saline), fixed in 11% glutaraldehyde, stained with a Crystal-Violet solution and analysed by microscopy. The scattering activity is evaluated observing the morphology of the colonies, which are clustered in the negative control (non-stimulated cells or stimulated with supernatant containing no factors) whereas they are dispersed in the positive control (HGF-His). The morphology of the cells themselves also varies upon stimulation: in fact, as it can be observed in Fig. 6, cells stimulated with HGF-His and Metron Factor-1 have a more oblong, spindle-shaped form, characterised by protrusions of the cell membrane called pseudopodes. These morphological variations are the consequence of factor-induced activation of a genetic program involving the modification of a series of cellular parameters, such as digestion of cell matrix by specific proteases and increase in motility.

The Table summarises the results of different tests, obtained with factors HGF, NK2-HGF, Metron Factor-1 and Magic Factor-1 on MDCK cells. The scattering units reported indicate the maximum dilution of the conditioned supernatant containing the factor, at which motogenic activity could be observed. Values are normalised for the protein content determined by western blotting as described above (see Fig. 5). These data indicate that the hybrid factors Metron Factor-1 and Magic Factor-1 have a scattering activity approximately three magnitudes higher than that of the NK2-HGF-His truncated form and one magnitude higher than that of HGF-His parental factor.

	HGF-his	NK2-his	Metron F-1	Magic Factor-1
Scatter units	900 ± 29	6 ± 5	5500 ± 1532	7600 ± 150

Table. Scattering activity of factors HGF-His, NK2-HGF-His Metron Factor-1 measured on kidney epithelial cells (MDCK). The scattering units reported indicate the maximum dilution of the conditioned supernatant containing the factor, at which a motogenic activity can be observed. Values are normalised for the protein content determined by western blotting.

Example 4a: Test for the evaluation of protection against programmed cell death (apoptosis).

One of the most characterised side effect of the chemotherapeutic drug cisplatin is the induction of programmed cell death (apoptosis) of epithelial cells of the proximal tubule, which leads to acute renal failure (ARF). Thus, a factor that protects against cisplatin-induced cytotoxicity is highly desirable. An in vitro functional test has been used, which allows to evaluate the percentage of cisplatin-treated apoptotic cells in the presence or in the absence of a survival factor. This system utilises a cell line (LOC) derived from epithelial cells of human kidney proximal tubule, immortalised by ectopic expression of SV40 large T antigen. For the functional test, cells are plated at day 0 in 96-well plates (10^3 cells/well) in 90% DMEM - 10% bovine calf serum. At day 1, the medium is substituted with medium containing 0.5% bovine calf serum buffered with 50 mM HEPES pH 7.4, which is added with different dilutions of the supernatant containing the recombinant factor. Cells are pre-incubated with these

factors for 6 hours, and then further incubated in the presence of 10 µg/ml cisplatin. At day 2, cells are washed with DPBS and the percentage of apoptotic cells is evaluated by the TUNEL technique (Boehringer Mannheim). The same kind of tests can be performed using primary cultures of human epithelial cells of kidney proximal tubule (PTEC). These tests proved that Metron Factor-1 and Magic Factor-1 have protecting activity against cisplatin-induced programmed cell death.

Example 4b: Protection against cisplatin-induced cytotoxicity by transient gene delivery of Metron Factor-1 and Magic Factor-1

The protective effect of Metron F-1 and Magic F-1 against cisplatin-induced cytotoxicity was further demonstrated by a transient gene delivery approach. Simian kidney epithelial cells (COS) were transfected with a control empty vector, an expression vector for Metron F-1, or an expression vector for Magic F-1. Following transfection, cells were treated for 16 hours with cisplatin (20 µg/ml) and the percentage of surviving cells in each transfection was determined. Cisplatin treatment was calibrated to cause the death of approximately 20% of the cells in the negative control. Ectopic expression of Metron F-1 or Magic F-1 increased the survival rate to about 92.3% and 94.0%, respectively.

Example 5: Activation of the Met receptor by Metron Factor-1 and Magic Factor-1

The ability of Metron Factor-1 and Magic Factor-1 to activate the Met receptor was tested by analysing the ability of the recombinant factors to induce tyrosine phosphorylation of Met in human epithelial cells (A549). For this analysis, A549 cells at 90% confluence in a 100 mm petri

dish were stimulated for 10 minutes with 1 ml of conditioned supernatant containing Metron Factor-1, Magic Factor-1 or no factor (as negative control) diluted 1:2.5 or 1:10 in DMEM. After stimulation, cells were washed in ice with PBS, lysated in 200 µl of lysis solution (1% Triton X-100, 5 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 7.4), added with a cocktail of protease inhibitors, immunoprecipitated for 2 hours at 4° C with 10 µl of Sepharose-A beads covalently conjugated with an anti-Met monoclonal antibody (Naldini, L. et al., 1991, EMBO J. 10: 2867-2878), washed 3 times in the same lysis solution, and heated at 90°C for 2 minutes to elute the absorbed proteins. These were separated by SDS-PAGE on a 8% BIS-acrylamide gel, transferred onto a membrane (Hybond-C; Amersham) and analysed by western blot. A mouse monoclonal antibody against phosphotyrosine (UBI) diluted 1:10000 was used as primary antibody and an anti-mouse IgG ovine antibody conjugated with peroxidase (Amersham) was used as secondary antibody. The secondary antibody was detected by ECL (Amersham) following the protocol provided by the manufacturer. This analysis revealed that Metron F-1 and Magic F-1 potently activate the Met receptor (Fig. 7).

<u>Example 6</u>: Protection against chemotherapy-induced renal failure by Metron Factor-1 in vivo

Metron-F-1 was tested in a model of nephrotoxicity in Balb-c mice. The method used was substantially as described (Kawaida K et al., 1994, Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice, Proc. Natl. Acad. Sci. 91:4357-4361). Briefly, renal failure was induced in male Balb-c mice weighing 20-25 g by an i.v.

injection of 7.5 mg/kg of $HgCl_2$ (7 animals/group). Renal damage was assessed by analysis of Blood Urea Nitrogen (BUN) and by histological evaluation, 72 h after $HgCl_2$ injection. Metron-F-1 was dissolved in 0.2 M NaCl, containing 0.01% Tween 80 and 0.25% human serum albumin and administered i.v. (100 µg/kg in a posological volume of 6.6 ml/kg) 0.5 h before and 6, 12, 24, 36 and 48 h after $HgCl_2$ injection. Controls animals were treated with the same amount of vehicle according to the same scheme.

Metron-F-1 significantly prevented the onset of acute renal failure induced by $HgCl_2$, evaluated in terms of BUN (figure 8). BUN values were closely paralleled by the histological findings, scored by an independent investigator.

In the following sequence listing:

SEQ. ID. NO. 1: Magic F-1 DNA coding sequence;

SEQ. ID. NO. 2: Magic F-1 amino acid sequence;

SEQ. ID. NO. 3: Metron F-1 DNA coding sequence;

SEQ. ID. NO. 4: Metron F-1 amino acid sequence.

INS B11